

## PATENT COOPERATION TREATY


## PCT

## INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)



Applicant's or agent's file reference 36435 PC 01	<b>FOR FURTHER ACTION</b>		See Form PCT/IPEA/416
International application No. PCT/DK2005/000131	International filing date (day/month/year) 25.02.2005	Priority date (day/month/year) 26.02.2004	
International Patent Classification (IPC) or national classification and IPC INV. C12Q1/68			
Applicant THOMSEN BIOSCIENCE AS et al.			
<p>1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of 10 sheets, including this cover sheet.</p> <p>3. This report is also accompanied by ANNEXES, comprising:</p> <p>a. <input checked="" type="checkbox"/> sent to the applicant and to the International Bureau a total of 1-4 sheets, as follows:</p> <p style="margin-left: 40px;"><input checked="" type="checkbox"/> sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).</p> <p style="margin-left: 40px;"><input type="checkbox"/> sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.</p> <p>b. <input type="checkbox"/> (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in electronic form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).</p>			
<p>4. This report contains indications relating to the following items:</p> <p><input checked="" type="checkbox"/> Box No. I Basis of the report</p> <p><input type="checkbox"/> Box No. II Priority</p> <p><input type="checkbox"/> Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p><input type="checkbox"/> Box No. IV Lack of unity of invention</p> <p><input checked="" type="checkbox"/> Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p><input type="checkbox"/> Box No. VI Certain documents cited</p> <p><input type="checkbox"/> Box No. VII Certain defects in the international application</p> <p><input type="checkbox"/> Box No. VIII Certain observations on the international application</p>			
Date of submission of the demand  22.12.2005		Date of completion of this report  12.05.2006	
Name and mailing address of the international preliminary examining authority:  European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016		Authorized officer  Reuter, U  Telephone No. +31 70 340-1036	



**INTERNATIONAL PRELIMINARY REPORT  
ON PATENTABILITY**

International application No.  
PCT/DK2005/000131

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**Box No. I Basis of the report**

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1. With regard to the **language**, this report is based on

- ☒ the international application in the language in which it was filed
- ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:
  - ☐ international search (under Rules 12.3(a) and 23.1(b))
  - ☐ publication of the international application (under Rule 12.4(a))
  - ☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a))

2. With regard to the **elements\*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report)*:

**Description, Pages**

1-41 as originally filed

**Claims, Numbers**

1-29 filed with the demand

**Drawings, Sheets**

1/2, 2/2 as originally filed

- ☒ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:

- ☐ the description, pages
- ☐ the claims, Nos.
- ☐ the drawings, sheets/figs
- ☐ the sequence listing (*specify*):
- ☐ any table(s) related to sequence listing (*specify*):

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

- ☐ the description, pages
- ☐ the claims, Nos.
- ☐ the drawings, sheets/figs
- ☐ the sequence listing (*specify*):
- ☐ any table(s) related to sequence listing (*specify*):

\* If item 4 applies, some or all of these sheets may be marked "superseded."

**INTERNATIONAL PRELIMINARY REPORT  
ON PATENTABILITY**

International application No.  
PCT/DK2005/000131

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**Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

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1. Statement

Novelty (N)	Yes: Claims	2-13,15-21,23-25,27-29
	No: Claims	1,14,22,26
Inventive step (IS)	Yes: Claims	
	No: Claims	1-29
Industrial applicability (IA)	Yes: Claims	1-29
	No: Claims	

2. Citations and explanations (Rule 70.7):

**see separate sheet**

**INTERNATIONAL PRELIMINARY REPORT  
ON PATENTABILITY**

International application No.  
PCT/DK2005/000131

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**Supplemental Box relating to Sequence Listing**

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**Continuation of Box I, item 2:**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this report was established on the basis of:
    - a. type of material:
      - ☒ a sequence listing
      - ☐ table(s) related to the sequence listing
    - b. format of material:
      - ☒ on paper
      - ☒ in electronic form
    - c. time of filing/furnishing:
      - ☐ contained in the international application as filed
      - ☐ filed together with the international application in electronic form
      - ☒ furnished subsequently to this Authority for the purposes of search and/or examination
      - ☐ received by this Authority as an amendment\* on
  2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table(s) relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
  3. Additional comments:
- \* *If item 4 in Box No. 1 applies, the listing and/or table(s) related thereto, which form part of the basis of the report, may be marked "superseded."*

**1    Re Item V**

**Reasoned statement with regard to novelty, inventive step or industrial applicability;  
citations and explanations supporting such statement**

The following documents are referred to in this communication:

- D1: OROU A ET AL: "Automatic separation of two PCRs in one tube by annealing temperature" TRENDS IN GENETICS, ELSEVIER, AMSTERDAM, NL, vol. 11, no. 4, April 1995, p. 127-128
- D2: HERRMANN B ET AL: "Detection of Neisseria gonorrhoeae from air-dried genital samples by single-tube nested PCR." JOURNAL OF CLINICAL MICROBIOLOGY. OCT 1996, vol. 34, no. 10, October 1996, p.2548-2551
- D3: TILSTON P ET AL: "A single tube nested PCR for the detection of hepatitis C virus RNA." JOURNAL OF VIROLOGICAL METHODS. MAY 1995, vol. 53, no. 1, May 1995, p. 121-129
- D4: ERLICH H A ET AL: "RECENT ADVANCES IN THE POLYMERASE CHAIN REACTION" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 252, no. 5013, 21 June 1991, p.1643-1651
- D5: KIM ET AL: "Detection of Bacillus anthracis using a Nested PCR Method" J KOREAN SOC MICROBIOL, vol. 33, no. 6, 1998, p.583-588,
- D6: JACKSON P J ET AL: "PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: the presence of multiple Bacillus anthracis strains in different victims." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA. 3 FEB 1998, vol. 95, no. 3, 3 February 1998, p.1224-1229
- D7: WO 03/059881 A (UNIVERSITY OF STRATHCLYDE; KHALAF, ABEDAWN; WAIGH, ROGER; SUCKLING, CO) 24 July 2003 (2003-07-24)
- D8: WO 01/12852 A (PE CORPORATION) 22 February 2001 (2001-02-22)
- D9: WO 98/02582 A (GEN-PROBE INCORPORATED) 22 January 1998 (1998-01-22)
- D10: WO 02/12263 A (ROCHE DIAGNOSTICS GMBH; F.HOFFMANN-LA ROCHE AG; SEELA, FRANK; BERGMANN) 14 February 2002 (2002-02-14)
- D11: BEYER W ET AL: "A nested PCR method for the detection of Bacillus

anthracis in environmental samples collected from former tannery sites."  
MICROBIOLOGICAL RESEARCH. MAY 1995, vol. 150, no. 2, May 1995,  
p.179-186

## 2 NOVELTY (Article 33(2) PCT)

### INDEPENDENT CLAIM 1:

- 2.1 D1 discloses a method of amplifying, and detecting, a target nucleic acid sequence, the method comprising the steps of (cf. D1, the whole document):
- a) providing a sample that may or may not comprise a target nucleic acid sequence,
  - b) providing a pair of outer primers and a pair of inner primers, a nucleic acid polymerase and standard reagents for PCR, the melting temperature ( $T_m$ ) of the pair of outer primers being at least 2°C higher than the  $T_m$  of the pair of inner primers ,
  - c) contacting the sample with the pair of outer primers and the pair of inner primers, and standard reagents for PCR, thus obtaining the reaction mixture,
  - d) cycling, at least two times, the temperature of the reaction mixture between a first denaturation temperature, a first annealing temperature and a first extension temperature, the first annealing temperature being similar to or lower than the lowest  $T_m$  of the outer primer pair and higher than the highest  $T_m$  of the inner primer pair,
  - e) cycling, at least two times, the temperature of the reaction mixture between a second denaturation temperature, a second annealing temperature and a second extension temperature, the second annealing temperature being similar or lower than the lowest  $T_m$  of the inner primer pair,
  - f) analysing the product of step d) and/or step e) to detect the presence of the target nucleic acid sequence.
- 2.2 A method according to independent claim 1 is also disclosed in D2 (cf. p.2549, col.1, par.3 and 4), D3 (cf. p.125, par.2) and D4 (cf. p.1645, col.1, par.3 to col.2, par.1 and Fig.1).
- Therefore, the subject-matter of **independent claim 1 is not novel** over D1 to D4 (Article 33(2) PCT).

INDEPENDENT CLAIM 14:

- 2.3 A kit comprising a pair of outer primers and a pair of inner primers, the melting temperature ( $T_m$ ) of the pair of outer primers being higher than the  $T_m$  of the pair of inner primers is disclosed in D1 (cf. whole document), D2 (cf. p.2549, col.1, par.3 and 4), D3 (cf. p.125, par.2) and D4 (cf. p.1645, col.1, par.3 to col.2, par.1 and Fig.1) and D5 (cf. tab.1 and abstract).

Therefore, the subject-matter of **independent claim 14 is not novel** over D1 to D5 (Article 33(2) PCT).

DEPENDENT CLAIM 22:

- 2.4 A kit for detection of *Bacillus anthracis*, the kit comprising a pair of outer primers and a pair of inner primers, the melting temperature ( $T_m$ ) of the pair of outer primers being higher than the  $T_m$  of the pair of inner primers, wherein the pair of outer primers and the pair of inner primers are selected from the pXO1 and pXO2 plasmid of *Bacillus anthracis*, is disclosed D5 (cf. tab.1 and abstract).

Therefore, the subject-matter of **dependent claim 22 is not novel** over D5 (Article 33(2) PCT).

INDEPENDENT CLAIM 26:

- 2.5 An analysis system for detection of a microorganism, the analysis system comprising a pair of outer primers and a pair of inner primers, the melting temperature ( $T_m$ ) of the pair of outer primers being higher than the  $T_m$  of the pair of inner primers, is disclosed in D1 (cf. whole document), D2 (cf. p.2549, col.1, par.3 and 4), D3 (cf. p.125, par.2), D4 (cf. p.1645, col.1, par.3 to col.2, par.1 and Fig.1) and D5 (cf. tab.1 and abstract).

Therefore, the subject-matter of **independent claim 26 is not novel** over D1 to D5 (Article 33(2) PCT).

3 INVENTIVE STEP (Article 33(3) PCT)

INDEPENDENT CLAIM 10:

- 3.1 Document D6 is considered to represent the most relevant state of the art for claim 10 in its present form. D6 discloses a method for detection of *Bacillus anthracis*, the

method comprising detecting a target nucleic acid sequence by nested PCR, the target nucleic acid sequence being specific for the pXO1 or pXO2 plasmid of *Bacillus anthracis*, wherein the pair of outer primers and the pair of inner primers are selected from pXO1 or pXO2 plasmid of *Bacillus anthracis* (cf. p.1226, col.2, par.2, tab.2).

- 3.2 The subject-matter of claim 10 differs from the subject-matter disclosed in closest prior art document D6 in that (a) the melting temperature ( $T_m$ ) of the pair of outer primers is at least 2°C higher than the  $T_m$  of the pair of inner primers and that (b) the temperature of the reaction mixture is cycled, at least two times, between a first denaturation temperature, a first annealing temperature and a first extension temperature, the first annealing temperature being similar to or lower than the lowest  $T_m$  of the outer primer pair and higher than the highest  $T_m$  of the inner primer pair, and than cycling, at least two times, the temperature of the reaction mixture between a second denaturation temperature, a second annealing temperature and a second extension temperature, the second annealing temperature being similar or lower than the lowest  $T_m$  of the inner primer pair.
- 3.3 The technical effect associated with said differences appears to be that the risk of amplification artefacts is reduced and the specificity of the nested PCR is increased.
- 3.4 The technical problem to be solved may therefore be regarded as providing an improved nested PCR for the detection of *Bacillus anthracis*. The proposed solution is to use (a) a pair of outer primers with a melting temperature ( $T_m$ ) that is at least 2°C higher than the  $T_m$  of the pair of inner primers and that (b) the temperature of the reaction mixture is cycled, at least two times, between a first denaturation temperature, a first annealing temperature and a first extension temperature, the first annealing temperature being similar to or lower than the lowest  $T_m$  of the outer primer pair and higher than the highest  $T_m$  of the inner primer pair, and than cycling, at least two times, the temperature of the reaction mixture between a second denaturation temperature, a second annealing temperature and a second extension temperature, the second annealing temperature being similar or lower than the lowest  $T_m$  of the inner primer pair.
- 3.5 This solution cannot be considered as involving an inventive step for the following



reasons:

- 3.5.1 It is well-known in the art that the specificity of nested PCR is increased by using an outer primer pair having a  $T_m$  that is higher than the  $T_m$  of the inner primer pair (cf. D1, whole document, D2, p.2549, col.1, par.3 and 4, D3, p.125, par.2 or D4, p.1645, col.1, par.3 to col.2, par.1 and Fig.1). Therefore, it would be obvious for the person skilled in the art searching for a way to improve the specificity of a nested PCR for detection of *Bacillus anthracis* to use a pair of outer primers with a  $T_m$  at least 2°C higher than the inner primer pair. This is especially the case as D5 describes a nested PCR for detecting *Bacillus anthracis*, wherein the outer primer pair has a  $T_m$  that is 12°C higher than the  $T_m$  of one of the inner primer pairs used (cf. D5, tab.1).
- 3.6 Hence, the subject-matter of **independent claim 10 appears not to involve an inventive step** (Article 33(3) PCT).
- 3.7 **Dependent claims 2 to 9, 11 to 13, 15 to 25, and 27-29** do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of novelty and/or inventive step:
- 3.7.1 It is known in the art that the  $T_m$  of primers can be increased by introducing modified nucleotides, PNA or LNA sequences into said primers (cf. D7 to D10, whole documents). Hence, the subject-matter of dependent claims 3 to 9 and 16 to 21 appears not to involve an inventive step.
- 3.7.2 The use of primers specific for the capA, capB, capC or lef gene for the detection of *Bacillus anthracis* by nested PCR is described in the prior art (cf. D6 and D11). Hence, the subject-matter of dependent claims 11 to 13 and 23 to 25 appears not to involve an inventive step.
- 3.7.3 The additional features of claim 28 and 29 fall within the scope of routine laboratory practise. Therefore, the subject-matter of dependent claims 28 and 29 appears not to involve an inventive step.
- 3.7.4 Also dependent claims 2, 15 and 27 do not appear to contain any additional

features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step. The technical features of said claims merely describe some of several straightforward possibilities and routine experimental optimization procedures from which the person skilled in the art would select in accordance with the circumstances, without the exercise of inventive skill, in order to solve the problem posed:

3.7.5 Given the fact that in the single tube nested PCR method disclosed in D3 the  $T_m$  of the outer primers is 10 and 12 °C (Wallace's rule: 2°C per G/C 2°C per A/T) higher than the  $T_m$  of the pair of inner primers (p. 123), the use of primers with 10 and 10 °C difference as claimed in claim 2 cannot be regarded as forming the basis of an inventive step for the reasons given above. The same applies to the subject matter of claims 15 and 27.

3.7.6 In the example disclosed in the application the difference of said melting temperatures is 9.9 and 13,5 °C.

3.7.7 The absolute value of the melting temperature used during the amplification is rather dependent on the value of the highest melting temperature itself than on the difference of the melting temperatures of inner and outer primers.

3.8 Claims 1-29 do not fulfil the requirement of inventive step of Art. 33(3) PCT.

36435PC01

1

PCT publication no.: WO 2005/083 114

Title: Method, Kit and System for enhanced nested PCR

Applicant: Thomsen Bioscience A/S

P&amp;V reference: 36435PC01

5

Response to first Written Opinion dated 1 August 2005

**AMENDED CLAIMS**

10

1. A method of amplifying, and optionally also detecting, a target nucleic acid sequence, the method comprising the steps of:

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a) providing a sample that may or may not comprise a target nucleic acid sequence,

20

b) providing a pair of outer primers and a pair of inner primers, a nucleic acid polymerase and standard reagents for PCR, the melting temperature ( $T_m$ ) of the pair of outer primers being at least 2 °C higher than the  $T_m$  of the pair of inner primers,

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c) contacting the sample with the pair of outer primers and the pair of inner primers, and standard reagents for PCR, thus obtaining the reaction mixture,

30

d) cycling, at least two times, the temperature of the reaction mixture between a first denaturation temperature, a first annealing temperature and a first extension temperature, the first annealing temperature being similar to or lower than the lowest  $T_m$  of the outer primer pair and higher than the highest  $T_m$  of the inner primer pair,

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e) cycling, at least two times, the temperature of the reaction mixture between a second denaturation temperature, a second annealing temperature and a second extension temperature, the second annealing temperature being similar to or lower than the lowest  $T_m$  of the inner primer pair,

f) optionally, analysing the product of step d) and/or step e) to detect the presence of the target nucleic acid sequence.

40 2. The method according to claim 1, wherein the  $T_m$  of the pair of outer primers is 2-10 °C higher than the  $T_m$  of the pair of inner primers.

36435PC01

2

3. The method according to claim 1, wherein at least one primer of the outer primer pair comprises a Tm-increasing component.
4. The method according to any of the claims 1 - 3, wherein both of the primers of the  
5 outer primer pair comprise a Tm-increasing component.
5. The method according to claim 4, wherein the Tm-increasing component binds non-specifically to nucleic acids.
- 10 6. The method according to any of claims 3-5, wherein the Tm-increasing component comprises one or more moieties selected from the group consisting of a modified nucleotide and a minor groove binding agent.
7. The method according to claim 6, wherein the modified nucleotide is a peptide nucleic  
15 acid (PNA) or a locked nucleic acid (LNA).
8. The method according to any of claims 3-7, wherein the Tm-increasing component increases the Tm of the primer with at least 1°C relative to the Tm of the same primer not comprising the Tm-increasing component.
- 20 9. The method according to any of the preceding claims, wherein the second denaturation temperature is at least 1°C lower than the first denaturation temperature.
10. A method for detection of *Bacillus anthracis*, the method comprising detecting a target  
25 nucleic acid sequence according to the method of claim 1-9, the target nucleic acid sequence being specific for the pXO1 or pXO2 plasmid of *Bacillus anthracis*, wherein the pair of outer primers and the pair of inner primers are selected from the pXO1 or pXO2 plasmid of *Bacillus anthracis*.
- 30 11. The method according to claim 10, wherein the pair of outer primers and the pair of inner primers are selected so as to amplify a target nucleic acid sequence related to a gene selected from the group of *B. anthracis* genes consisting of capA gene, the capB gene, the capC gene and the lef gene.
- 35 12. The method according to claim 10 or 11, wherein target nucleic acid sequence is related to the capA gene and
- a primer of the pair of outer primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 1, SEQ ID NO: 2, a homologous sequence thereof, and a complementary sequence thereof, and
  - 40 - a primer of the pair of inner primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 3, SEQ ID NO: 4, a homologous sequence thereof, and a complementary sequence thereof.

36435PC01

3

13. The method according to any of the claims 10-12, wherein target nucleic acid sequence is related to the *capA* gene and the pair of outer primers comprises SEQ ID NOs: 1 and 2 and/or the pair of inner primers comprises SEQ ID NOs: 3 and 4.

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14. A kit comprising a pair of outer primers and a pair of inner primers, the melting temperature ( $T_m$ ) of the pair of outer primers being higher than the  $T_m$  of the pair of inner primers.

10 15. The kit according to claim 14, wherein the  $T_m$  of the pair of outer primers is 2-10 °C higher than the  $T_m$  of the pair of inner primers.

16. The kit according to claim 14 or 15, wherein at least one primer of the outer primer pair comprises a  $T_m$ -increasing component.

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17. The kit according to claim 16, wherein both of the primers of the outer primer pair comprises a  $T_m$ -increasing component.

18. The kit according to any of the claims 16-17, wherein the  $T_m$ -increasing component  
20 binds non-specifically to nucleic acids.

19. The kit according to any of the claims 16-18, wherein the  $T_m$ -increasing component comprises one or more moieties selected from the group consisting of a modified nucleotide and a minor groove binding protein.

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20. The kit according to claim 19, wherein the modified nucleotide is a peptide nucleic acid (PNA) or a locked nucleic acid (LNA).

21. The kit according to any of the claims 16-20, wherein the  $T_m$ -increasing component  
30 increases the  $T_m$  of the primer with at least 1°C relative to the  $T_m$  of the same primer not comprising the  $T_m$ -increasing component.

22. A kit according to any of the claims 14-21 for detection of *Bacillus anthracis*, the kit comprising a pair of outer primers and a pair of inner primers, the melting temperature  
35 ( $T_m$ ) of the pair of outer primers being higher than the  $T_m$  of the pair of inner primers, wherein the pair of outer primers and the pair of inner primers are selected from the pXO1 or pXO2 plasmid of *Bacillus anthracis*.

40 23. The kit according to claim 22, wherein the pair of outer primers and the pair of inner primers are selected so as to amplify a target nucleic acid sequence within a gene selected from the group of *B. anthracis* genes consisting of *capA* gene, the *Cap B* gene, the *Cap C* gene, the *lef* gene.

36435PC01

4

24. The kit according to claim 22 or 23, wherein target nucleic acid sequence is related to the capA gene and

- a primer of the pair of outer primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 1, SEQ ID NO: 2, a homologous sequence thereof, and a complementary sequence thereof, and
- a primer of the pair of inner primers comprises a nucleic acid sequence selected from the group of SEQ ID NO: 3, SEQ ID NO: 4, a homologous sequence thereof, and a complementary sequence thereof.

10 25. The kit according to any of the claims 22-24, wherein target nucleic acid sequence is related to the capA gene and the pair of outer primers comprises SEQ ID NOs: 1 and 2 and/or the pair of inner primers comprises SEQ ID NOs: 3 and 4.

15 26. An analysis system for detection of a microorganism, the analysis system comprising a pair of outer primers and a pair of inner primer, the melting temperature ( $T_m$ ) of the pair of outer primers being higher than the  $T_m$  of the pair of inner primers.

27. The analysis system according to claim 26, wherein the  $T_m$  of the pair of outer primers is 2-10 °C higher than the  $T_m$  of the pair of inner primers.

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28. The analysis system according to claim 26 or 27, wherein at least one primer of the outer primer pair comprises a  $T_m$ -increasing component.

25 29. The analysis system of any of the claims 26-28, wherein the analysis system is selected from the group consisting of a lateral flow device, a biochip, and a microarray.